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Comparative Analysis of Whole Blood Lysis Methods for Flow Cytometry

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We performed a parallel evaluation of six whole blood lysis methods comparing light scatter and quantitative fluorescence intensity based on quantitative flow cytometry, of selected lymphocyte subsets and CD34+ cells. Leukocytes prepared with FACS Lysing Solution (BDIS), Immunolyse (Coulter) and Optilyse B (Immunotech) consistently gave lower forward scatter values than those prepared with ACK (BioWhitaker), Ortho-mune (Ortho) and ImmunoPrep (Coulter). Debris, defined as CD45 negative events with the threshold off, accounted for ~80% of all events with ACK and Ortho-mune. The other lysing methods consistently yielded less debris (~50%) with Immunolyse generating only ~16% debris. Optilyse and FACS lyse consistently displayed the lowest percentage of lymphoid cells (CD45+/CD14−) in the three part differential. The percentage of CD3+, CD20+, CD5+, and CD16/CD56+ cells was consistent with all methods but CD4 and CD8 determinations showed inconsistent variation with ACK and Ortho-mune. In addition, the fluorescence intensity of CD14 PE and CD8 PE staining was markedly decreased on cells prepared with ImmunoPrep. Finally, the clearest separation of CD34+ cells was observed with ACK and Ortho-mune. Our data demonstrate that the method used for red cell lysis can have definite impact on immunophenotyping and selected methods appear to be more suitable for specific applications. Cytometry 30:124–133, 1997. © 1997 Wiley-Liss, Inc.†

Key terms: flow cytometry; whole blood lysis; immunophenotyping; CD34

In clinical flow cytometry, whole blood lysis methods have substituted for density gradient separation of peripheral blood mononuclear cells as the routine preparation technique for immunophenotyping. This is a consequence of the decreased requirement for manipulation and blood volume with the former method resulting in less risk associated with handling of the blood and greater ease in obtaining samples from pediatric patients. Reports to date have mainly focused on a comparison between whole blood lysis and Hypaque-Ficoll density gradient (1–8) and only limited data are available on the parallel comparison of commercially available whole blood lysis methods. Carter et al. (9) evaluated four whole blood lysis methods and the Hypaque-Ficoll density gradient method on lymphocyte forward-angle scatter, autofluorescence and immunofluorescent staining for CD45, CD14, and CD13. However, these authors did not address the question whether the currently available methods for whole blood lysis affect 1) the fluorescence intensity and 2) the quantitation of the various lymphocyte subsets. Flow cytometric quantitation of lymphocyte subsets has become a pivotal tool in the clinical immunological evaluation of immune deficiencies or immune disorders (10). For example, it has been clearly established that monitoring of the CD4 T cell number has important prognostic implications for individuals with HIV infections (11,12). Moreover, during recent years, there has been an increased interest in quantitative flow cytometry to standardize measurement of fluorescence intensity (13,14).

We performed a side-by-side evaluation of six commercially available whole blood lysis methods comparing the physical light scatter properties, fluorescence intensity of directly conjugated monoclonal antibody staining using quantitative flow cytometry, and quantitation of the various lymphocyte subsets. In addition, we also studied the effects of the whole blood lysis methods on peripheral blood CD34+ cell determinations. This marker has been used to quantify the hematopoietic precursor population and the flow cytometric techniques augment and may even replace the time-consuming clonogenic assay (15,16). Such determinations are important in patients recovering from chemotherapy, as well as those undergoing autologous, allogeneic, and cord blood stem cell transplantation.

Our results show that there are marked differences between the commercially available whole blood lysis

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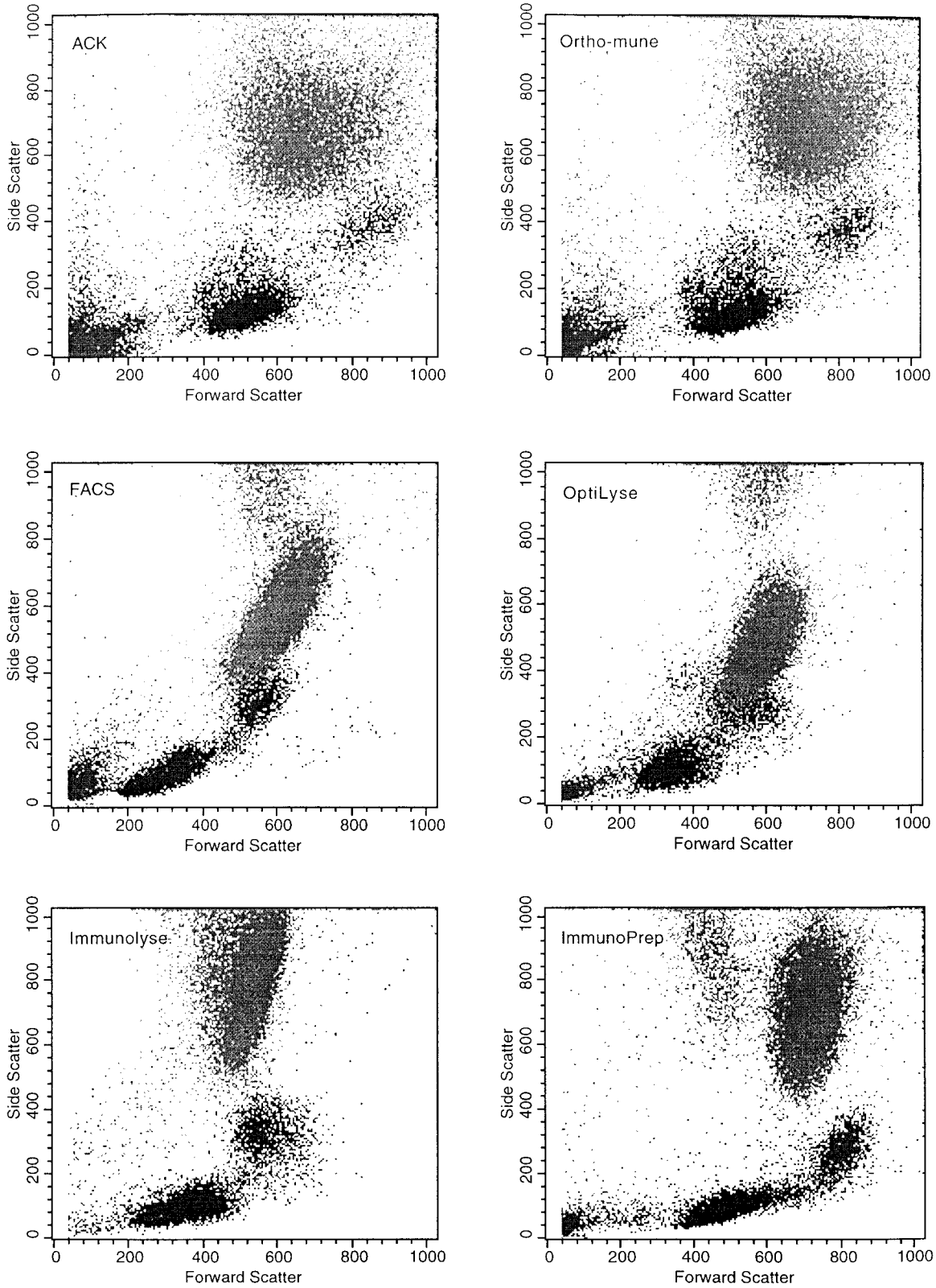

FIG. 1. Effect of whole blood lysis method on light scatter properties of nucleated peripheral blood cells. After staining with CD14 and CD45, red blood cells were lysed using six different whole blood lysis methods (Materials and Methods). Threshold and photomultiplier tube settings were the same for the six different conditions and all events were acquired. The figure shows dot plots for linear FSC vs. linear SSC. The data are from one representative determination out of six studies on different individuals.

Table 1
Effect of Whole Blood Lysis Method on Percentage of Lymphocytes

	Lymphocyte percentage in normal subjects					
Lysing reagent	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
ACK	28	35	30	36	24	27
Ortho-mune	26	28	29	41	25	32
ImmunoPrep	27	31	29	36	21	43
FACS-Lyse	24	25	25	33	19	28
Optilyse B	25	23	24	34	13	30
Immunolyse	29	29	26	37	21	33
CellDyn 3500	30	33	36	37	28	38

Each column reports the lymphocyte percentage for a normal subject obtained by the three part flow cytometric differential with the six different lysing methods as well as the automated hematology instrument (CellDyn 3500).

methods with regard to the physical light scatter properties, generation of non-leucocyte debris, distribution of the three major leucocyte populations, and the fluorescence intensity of directly conjugated monoclonal antibody staining. In addition, under certain circumstances whole blood lysis methods can affect quantitation of selected lymphocyte subsets.

MATERIALS AND METHODS

Sample Preparation

Whole blood specimens (6 ml) from normal donors were collected by using EDTA as anticoagulant (Vacutainer tubes, Becton Dickinson, San Jose, CA). The blood was processed immediately and a white blood count with differential was obtained (CellDyn 3500, Abbott, Chicago, IL).

The lysing reagents were evaluated based on a common protocol. A 2- to 3-ml aliquot of blood was diluted 1:5 to 1:7 with phosphate-buffered saline (PBS) (Quality Biological, Gaithersburg, MD), centrifuged at 1,000 rpm (Beckman JS-4.0 Horizontal Rotor, Beckman Instruments, Inc., Palo Alto, CA), and reconstituted to the original volume with PBS. This washing step eliminates any potential artifactual staining (17). Blood was stained with the monoclonal reagents (see below) for 10 min prior to lysing. Lysis was performed with ACK Lysing Buffer (ACK, BioWhittaker, Walkersville, MD), FACS Lysing Solution (FACS lyse, Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA), Optilyse B Lysing Solution (Optilyse, Immunotech, Marseille, France), Ortho-mune Lysing Reagent (Ortho-mune, Ortho Diagnostic Systems, Raritan, NJ), Immunolyse and ImmunoPrep Leucocyte Preparation System (ImmunoPrep, Coulter Immunology, Hialeah, FL). The amount of lysing reagent that was used and the time of lysing conformed to the manufacturer's recommended procedures. With FACS lyse, Ortho-mune and ACK, 2 ml of lysing solution was added to 200 µl blood. After a brief vortex, the tubes were incubated at room temperature for 10 min. The incubation with FACS lyse was performed in the dark. The lysing procedure with Optilyse consisted of two consecutive incubations of 10 min at room temperature. The first incubation of 10 min was with 100 µl of the lysing reagent, the composition of which is proprietary. For the second incubation, 2 ml deionized water was added. With Immunolyse, blood was incubated with 1 ml of the lysing reagent for 1 min. Thereafter, 250 µl of paraformaldehyde based fixing reagent, provided by the manufacturer, was added. With the Multi-Q-Prep system (ImmunoPrep), which is an automated system, three reagents—1) formic acid (lysing reagent), 2) sodium carbonate, sodium chloride and sodium sulfate (stabilizers), and 3) paraformaldehyde (fixative)—were added sequentially by the machine over a 35-s time period. All preparations were washed with PBS (once for FACS lyse and ImmunoPrep; twice for Immunolyse, Ortho-mune, ACK, Optilyse) until the preparations were free of red blood cells. Because preparations lysed with Immunolyse and ImmunoPrep were fixed during the lysing procedure, the final preparations were resuspended in PBS. Since ACK and Ortho-mune lysing reagents do not contain a fixative and since the composition of the FACS lyse and Optilyse lysing reagent is proprietary and the presence or concentration of a fixative was not known, the final preparations obtained with these reagents were resuspended in 200 µl PBS containing 1% paraformaldehyde. In this way, all preparations were washed according to manufacturer's instructions and fixed.

Preparations were stained with a combination of CD45-fluorescein isothiocyanate (FITC) and CD14-phycoerythrin (PE) (Leucogate [BDIS]); a combination of IgG1-FITC, IgG1-PE (Simultest Control [BDIS]) and IgG1-PerCP (BDIS); a combination of CD4-FITC/CD8-PE (Immunotech) and CD3-PerCP (BDIS); a combination of CD3-FITC, CD16-PE and CD56-PE (Simultest CD3/CD16 + 56 [BDIS]); a combination of CD20-FITC (BDIS) and CD5-PE (BDIS); CD34-FITC (BDIS); and CD 34-PE (BDIS).

Flow Cytometry

Flow cytometric analysis was performed using a FACScan (BDIS) flow cytometer with linear forward scatter (FSC) and side scatter (SSC) and log fluorescence 1 (FL1), fluorescence 2 (FL2) and fluorescence 3 (FL3) detection. Back-gating based on CD14/CD45 staining was used to determine the scatter characteristics of lymphocytes as previously described (18). An extended light scatter based lymphocyte gate was also used for CD34 determinations (19). Ten thousand events were collected for all antibody combinations and 100,000 events were collected for CD34 determinations. List mode data for light scatter and fluorescence intensity (530 nm, 585 nm, 677 nm) were collected for all tubes.

Fluorescence intensity measurements were standardized using fluorescent beads (QC3, Flow Cytometry Standards Corporation, San Juan, PR). With the compensation circuits off, the beads were placed in relative linear channel 626 for FL1, in relative linear channel 791 for FL2, and in relative linear channel 938 for FL3 by adjusting the photomultiplier tube settings. The compensation circuits for FL1 and FL2 were adjusted with FITC and PE Quantum 1000 Microbeads (Flow Cytometry Standards Corporation).

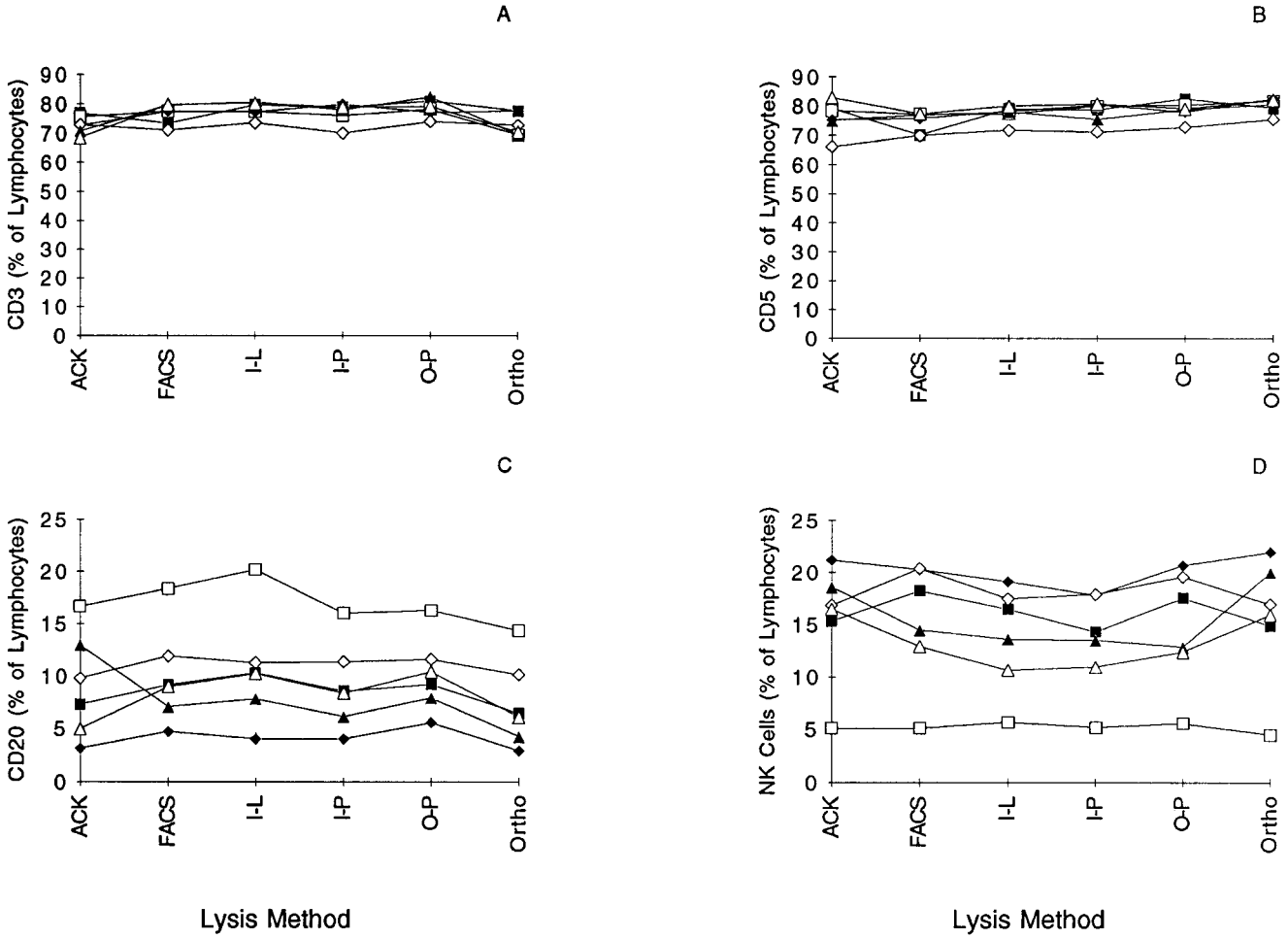

FIG. 2. Effect of whole blood lysis method on quantitation of lymphocyte subsets. Lymphocytes from six normal donors were stained with CD3 **(A),** CD5 **(B),** CD20 **(C),** and CD16 + CD56 **(D)** and prepared for flow cytometric analysis using six different whole blood lysis methods; ACK, FACS lyse (FACS), Immunolyse (I-L), ImmunoPrep (I-P), Opti-Lyse (O-L), and Ortho-mune (Ortho). Each normal donor is represented by a separate symbol. Positive staining cells are expressed as percentage of total lymphocyte population.

Quantitation of molecules of equivalent soluble fluorochrome (MESF) was performed as described previously (14). Calibration plots were constructed for the FL1 channel and the FL2 channel from the assigned values and mean peak channels of the Quantum 1000 fluorescent microbead populations by using the Quickcal program (Flow Cytometry Standards Corporation). For each calibration curve, the primary performance parameters for fluorescence intensity assessment were within the acceptable range as described by Schwartz et al. (14); i.e. the average residual percent $<3\%$, the detection threshold $<1{,}000$ MESF, the coefficient of response between 59 and 69, and the zero channel value <200 MESF.

Analysis of list mode data was performed using Cell Quest software (BDIS) and positive fluorescence was established based on the fluorescence specific isotype controls for each subject. The percentage positive cells for each antibody was determined as well as the MESF value calculated using the previously described calibration curves.

RESULTS

Effect of Whole Blood Lysis Method on the Physical Light Scatter Properties of Nucleated Peripheral Blood Cells and on the Amount of Debris

Figure 1 shows dot plots for FSC vs. linear SSC for the six different whole blood lysis methods for white blood cell enrichment. The same threshold and photomultiplier tube settings were used for all conditions in order to allow direct comparison. Major differences between the lysis methods were observed with regard to the light scatter properties of the different cell populations and the amount of debris generated by the lysing procedure. Lymphocytes, monocytes and granulocytes prepared with FACS lyse, Immunolyse and Optilyse consistently gave lower FSC values than those prepared with ACK, Ortho-mune and ImmunoPrep procedures, indicating that lysing with the former methods resulted in a relative alteration of the cells when compared to the latter methods. Lymphocytes and

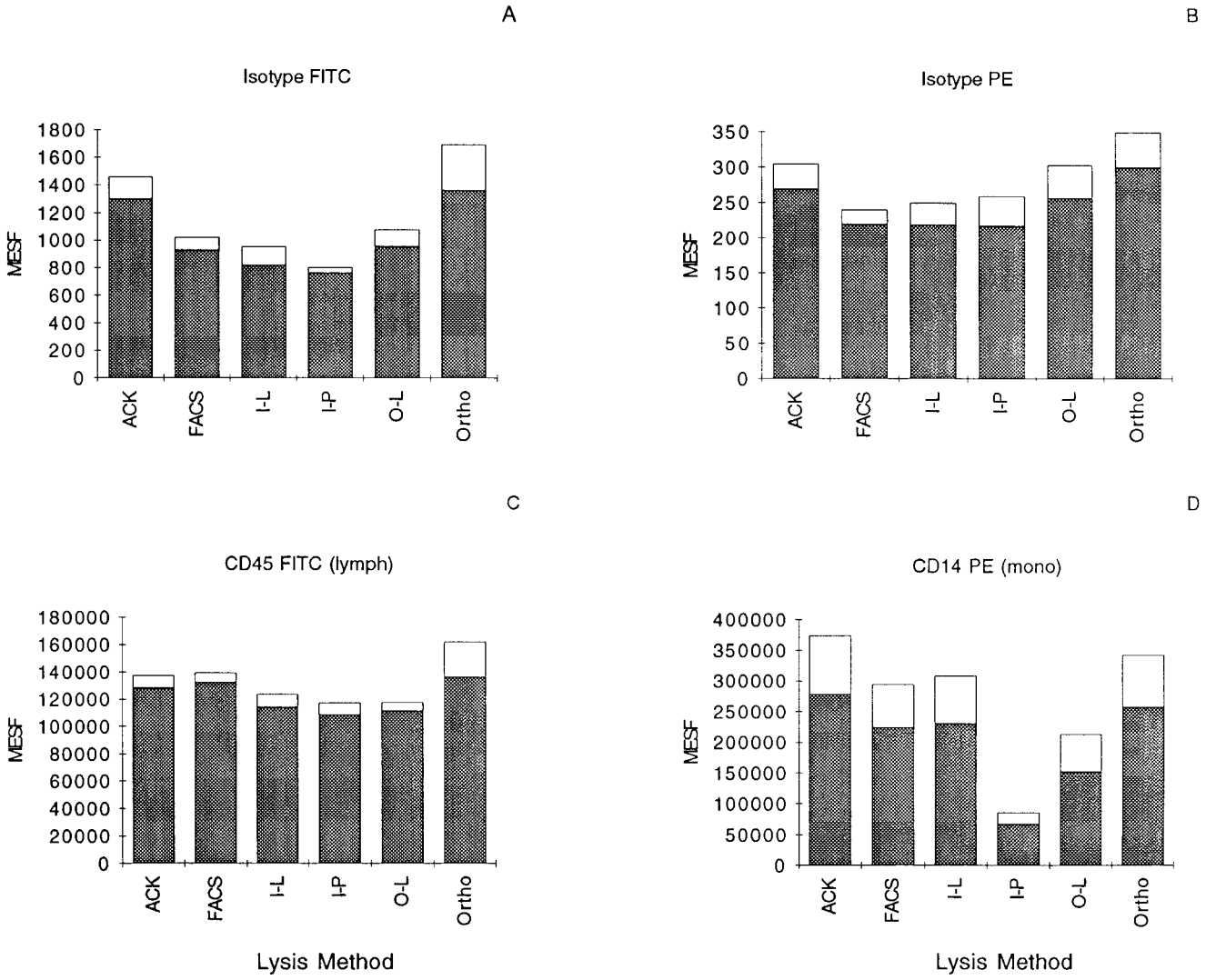

FIG. 3. Effect of whole blood lysis method on fluorescence intensity of immunofluorescent staining. Blood cells were stained with isotype control **(A and B),** CD45 **(C),** and CD14 **(D)** as described in Materials and Methods. MESF values were determined after whole blood lysis with six different whole blood lysis methods; ACK, FACS lyse (FACS), Immunolyse (I-L), ImmunoPrep (I-P), Opti-Lyse B (O-L), and Ortho-mune (Ortho). Data represent mean values (shaded bar) ± S.D. (open bar) for six independent determinations on the different normal blood donors performed on separate days.

monocytes prepared by Hypaque-Ficoll density gradient were found in the same FSC channels as those prepared with Ortho-mune and ACK (data not shown). The overall pattern of linear FSC vs. linear SSC found with ACK resembled the pattern found with Ortho-mune. Characteristic for these two lysing methods, both of which are based on hypotonic incubation with ammonium chloride, is that the cell populations, especially the granulocytes, are distributed over a large surface in two-dimensional FSC–SSC plots. With FACS lyse, Optilyse B, Immunolyse and ImmunoPrep the cell populations are compressed on FSC–SSC plots.

Figure 1 also indicates that whole blood lysis performed with ACK and Ortho-mune generated substantial amounts of debris, which is located in the lower left corner of the FSC–SSC plot (verified by back-gating from CD45/CD14 staining). Less debris was found with FACS lyse, Optilyse and ImmunoPrep, whereas only a small amount of debris was produced by Immunolyse. Because the threshold for FSC was set at channel 52 for the analyses presented in Figure 1, it was possible that in these experiments debris was selectively excluded. In order to detect all events and to quantify the amount of debris generated by the various lysing techniques, we repeated the experiment with the threshold set at channel 0 for FSC and SSC. Under these conditions (threshold off), debris defined as CD45 negative events averaged 81% ± 4% for Ortho-mune, 79% ± 5% for ACK, 54% ± 7% for Optilyse B, 53% ± 4% for FACS lyse, 50% ± 7% for ImmunoPrep, and 16% ± 1% for Immunolyse of all events collected.

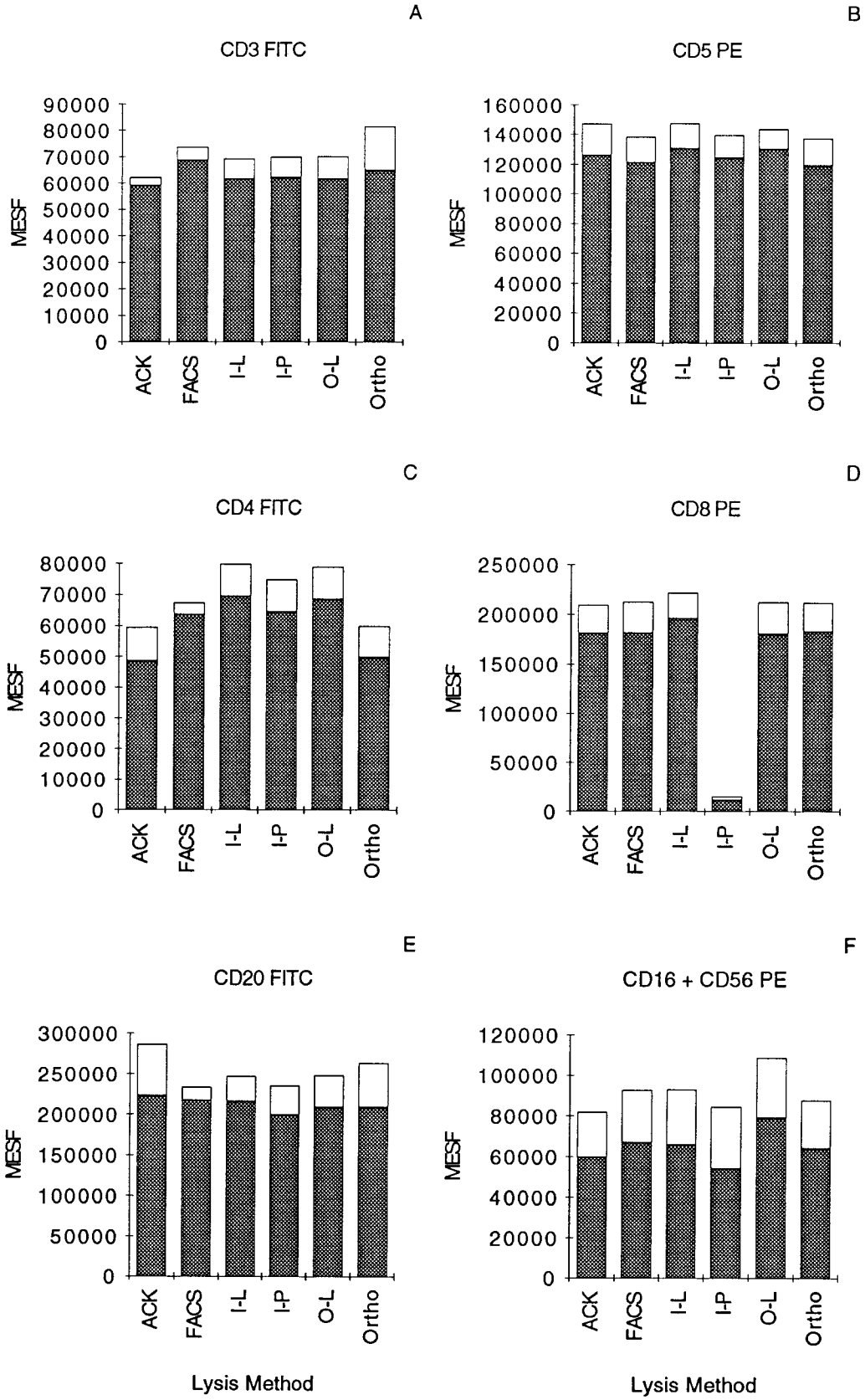

FIG. 4. Effect of whole blood lysis method on fluorescence intensity of immunofluorescent staining. Blood cells were stained with CD3, CD5, CD4, CD8, CD20, and CD56 + 16, as described in Materials and Methods. MESF values were determined after whole blood lysis with six different whole blood lysis methods; ACK, FACS lyse (FACS), Immunolyse (I-L), ImmunoPrep (I-P), Opti-Lyse B (O-L), and Ortho-Mune (Ortho). Data represent mean values ± S.D. for six independent determinations on the different normal blood donors.

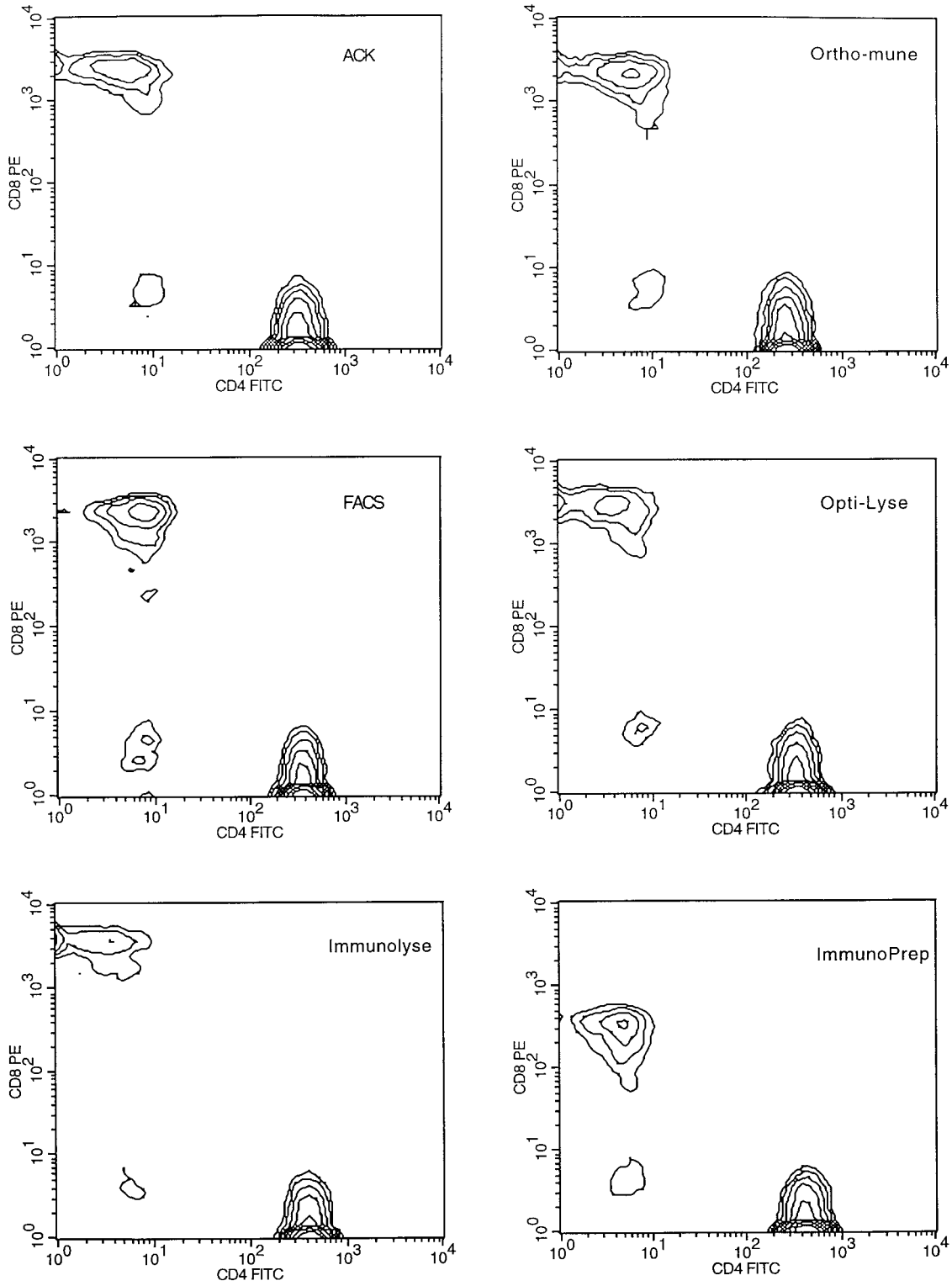

FIG. 5. Effect of whole blood lysis method on fluorescence intensity of CD8 and CD4. Contour plots of CD3+ CD4 and CD3+ CD8 T cells after whole blood lysis with six different methods. Data are from one of six representative experiments.

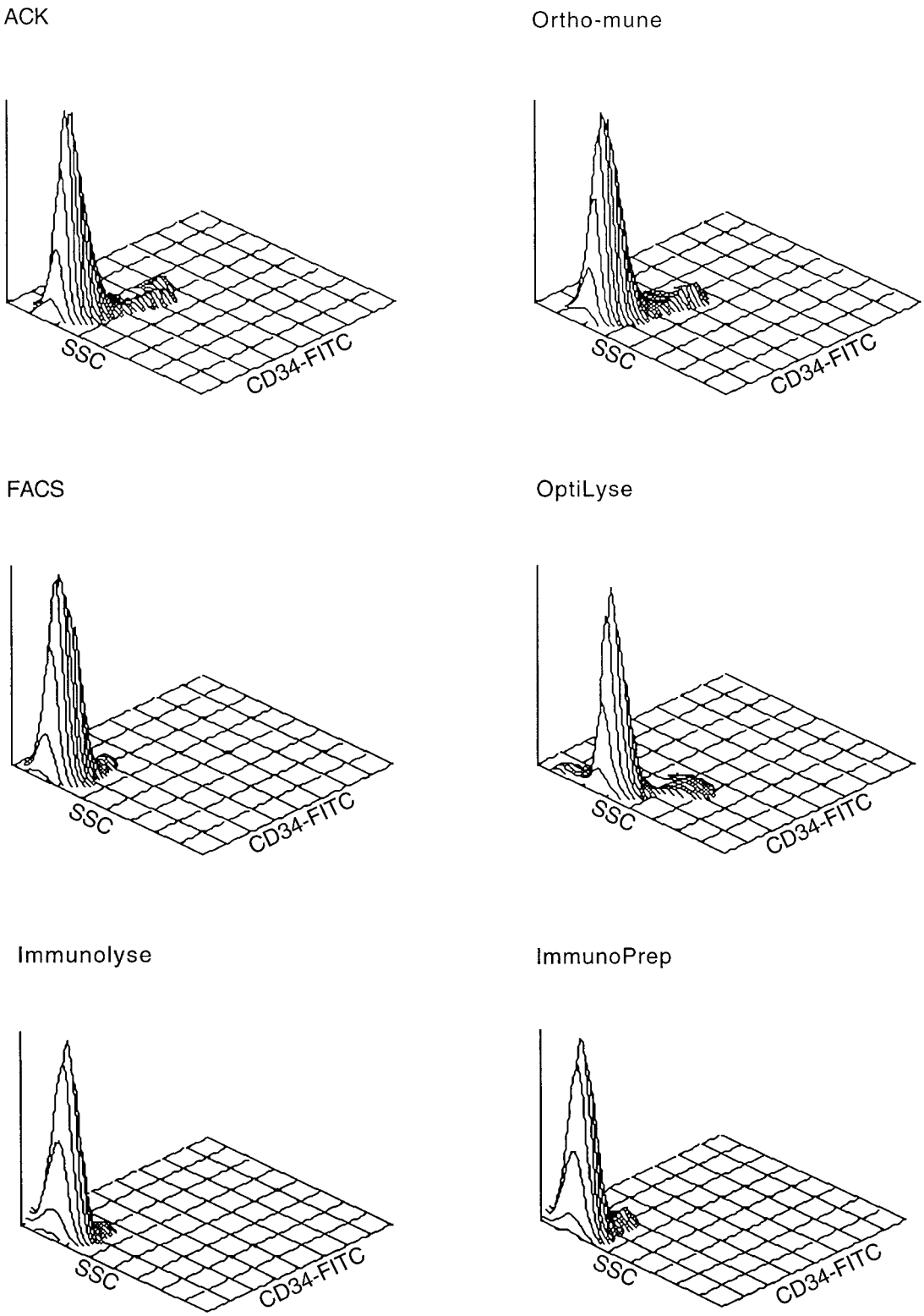

FIG. 6. Effect of whole blood lysis method on determination of CD34+ cells. CD34+ cells were determined as described in Materials and Methods using six different whole blood lysis methods. The figure represents isometric representations of CD34 FITC (x axis) vs. SSC (y axis). Data are from one representative of six independent determinations.

Table 2
Effect of Whole Blood Lysis Method on Enumeration of CD34+ Cells

	CD34 (cells/μl)	
Lysing reagent	CD34 FITC	CD34 PE
ACK	1.3 ± 0.5	1.4 ± 0.6
Ortho-mune	1.4 ± 0.4	1.4 ± 0.4
ImmunoPrep	1.0 ± 0.3*	1.3 ± 0.6
FACS-Lyse	0.6 ± 0.4*	0.7 ± 0.4*
Optilyse B	0.8 ± 0.3*	0.9 ± 0.4*
Immunolyse	0.8 ± 0.3*	0.9 ± 0.3*

CD34+ cells were determined as described in Materials and Methods using FITC- and PE-labeled antibodies. Values represent means ± SD of six independent determinations using six different normal donors.

*$P < 0.05$ when compared to Ortho-mune by paired two-tailed *t*-test.

Effect of Whole Blood Lysis Method on the Percentages of Positively Staining Cells

Cells from six normal donors were stained and lysed with the six different methods. Each donor was analyzed on a separate day. The percentage of lymphoid cells (CD45+/CD14−) was determined for each experiment. Preparations lysed with OptiLyse and FACS lyse consistently displayed the lowest percentage of lymphoid cells (Table 1). When compared to a white blood count and differential obtained with an automated hematology cell counter, OptiLyse B and FACS lyse consistently gave the lowest values for percentage of lymphoid cells.

Next, we investigated whether the percentage of positive cells after staining with CD3, CD5, CD20, and CD16 + CD56 was affected by the lysis method. As shown in Figure 2, no substantial differences were found between the various lysis methods, indicating that the quantitation of the major lymphocyte subsets, i.e. T cells, B cells and natural killer cells, was not influenced by the lysis method.

Effect of Whole Blood Lysis Method on Fluorescence Intensity of Immunofluorescent Staining

The effects of lysis methods on fluorescence intensity of immunofluorescent staining, quantitated by MESF, are summarized in Figure 3 and 4. Lymphocytes prepared by hypotonic lysis with ACK and Orthomune consistently showed the highest fluorescence intensity for FITC and PE isotype control (Fig. 3A and 3B) and the lowest fluorescence intensity for CD 4 FITC (Fig. 4C). Monocytes exhibited markedly lower fluorescence intensity with CD14 PE (Fig. 3D) and lymphocytes showed a log reduction in fluorescence intensity with CD8 PE (Fig. 4D) when prepared with ImmunoPrep. A representative contour plot demonstrating the marked decrease in CD8 PE fluorescence using ImmunoPrep is shown in Figure 5. No effects on fluorescence intensity were observed for the other lysing reagent-antibody combinations.

Effect of Whole Blood Lysis Method on CD34+ Cell Determination

The effects of whole blood lysis method on CD34+ cell determination are represented in Figure 6. The ammonium chloride based lysis methods, ACK and Orthomune, differed from the other tested methods (FACS lyse, Optilyse B, Immunolyse and ImmunoPrep) in two aspects. First, the former methods gave the best separation of CD34+ cells in the expanded lymphocyte gate using either CD34 FITC (Fig. 6) or CD34 PE (data not shown). Second, the number of CD34+ cells identified in this lymphocyte gate was significantly higher with ACK and Ortho-mune than with the other lysis reagents (Table 2).

DISCUSSION

Whole blood lysis serves as the standard preparative approach for immunophenotyping in most clinical laboratories. As such, it is imperative that any impact on results by the various methods available should be documented. We are unaware of a parallel comparison between the presently available commercial whole blood lysing methods and therefore performed this study.

The mechanism of action of red blood cell lysis with ACK and Ortho-mune is an accumulation of ammonium chloride within the cell. This accumulation increases the osmotic pressure within the cell resulting in a rupture of the red cell membrane. With ImmunoPrep, lysis is based on incubation with formic acid. The composition of FACS lyse is proprietary but involves diethylene glycol in the red cell lysing process. Likewise, Optilyse and Immunolyse are proprietary but the mechanism of action of these agents may involve saponin, a commonly used red cell lysing agent based on its detergent action.

Whole blood lysis with ACK and Ortho-mune differs from the other methods tested in various aspects. First, these two methods generate considerable amounts of debris. Debris, defined as CD45 negative events collected with the threshold off, approximated 80% of all events with both of these agents. The other methods yielded less debris with Immunolyse generating consistently ~16%. Second, lysing with ACK and Ortho-mune resulted in the highest fluorescence intensity for the isotype control (FITC and PE) and the lowest fluorescence intensity for CD4 FITC on lymphocytes. Third, our data suggest that ACK and Ortho-mune may introduce undesirable effects in the quantitation of CD4 and CD8 cells, at least in some control subjects. During our initial experiments, we found that, when compared to the other lysis methods, lysing with ACK and Ortho-mune resulted in a drastic decrease in percentage of CD4 T cells in two of six normal donors. In these individuals there was a corresponding increase in percentage of CD8 T cells. For both individuals, these effects were reproduced once in a repeat experiment (1 week later) but were not seen in a subsequent second repeat experiment (9 weeks later). The reason(s) for the shift in CD4/CD8 ratio after lysis with ACK and Ortho-mune in some studies of specific control subjects is unknown. Finally, despite the increased level of debris and

alterations in certain T cell markers, our data indicate that ACK and Ortho-mune may prove superior to the other tested whole blood lysis methods for quantitation of CD34+ cells. These methods gave the best separation and yield of CD34+ cells evaluated in an expanded lymphocyte gate.

Although no differences in quantitation of lymphocyte subsets were found with the Multi-Q-Prep automated technique (ImmunoPrep) when compared to the other methods tested, we found major changes with regard to the fluorescence intensity. Lymphocytes prepared by this method exhibited the lowest fluorescence intensity for CD8 and CD14 with almost a log reduction in the CD8 PE fluorescence. This effect on fluorescence intensity could prove to be a disadvantage with the ImmunoPrep in view of the growing interest in quantitating fluorescence intensity to monitor certain aspects of disease (13,20).

Lymphocytes, monocytes and granulocytes prepared with Optilyse, FACS lyse and Immunolyse gave lower forward scatter values than those prepared with ACK, Ortho-mune and ImmunoPrep. No substantial differences were seen between Optilyse, FACS lyse and Immunolyse with respect to quantitation of lymphocyte subsets and with respect to fluorescence intensity. However, of these three methods, lysing with Immunolyse 1) resulted in the best separation of the lymphocyte, granulocyte and monocyte population in FSC vs. SSC plot and 2) produced the lowest amount of debris.

During the course of the evaluation, DAKO Uti-Lyse (DAKO, Carpinteria, CA) became available and was tested according to the manufacturer's instructions on three normal donors using the same set-up as outlined in the Materials and Methods section. Insufficient lysing was obtained with the blood from one donor and the results from the two other donors were comparable to the other lysing methods except for the very low number of CD34+ cells detected.

In conclusion, our data demonstrate that the method used for the red blood cell lysis can have major impact on physical light scatter properties of leukocytes, the generation of non-leukocyte events, the fluorescence intensity of immunofluorescent staining, and the quantitation of lymphocyte subsets. Selected methods appear to be more suited for lymphocyte evaluation whereas other methods are more suited for CD34 determination. The optimal method of whole blood lysis method chosen should be determined for each specific flow cytometric application.

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